Circular Dichroism of C3a Anaphylatoxin

EFFECTS OF pH, HEAT, GUANIDINIUM CHLORIDE, AND MERCAPTOETHANOL ON CONFORMATION AND FUNCTION*

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SUMMARY

Circular dichroism spectra for C3a anaphylatoxins (protein fragments generated enzymatically in serum during activation of the third component of complement (C3)) from human and porcine sources were compared in the region of 190 to 250 nm. The spectra were indistinguishable in this region, although an estimated difference of approximately 20% exists between the primary structures of human and porcine C3a. Calculations indicated a total of 10 to 15% α-helical content based on either the 208 or 222 nm extremum of the CD spectra. Addition of either mercaptoethanol or 6 M guanidinium chloride to human C3a produced a marked decrease in the mean residue ellipticity centered at 222 nm without irreversibly affecting the biological activity. Simultaneous addition of mercaptoethanol and guanidinium chloride virtually eliminated the CD contribution at 222 nm and resulted in more than 90% inactivation of the anaphylatoxin. Removal of the denaturant and reducing agent restored both the biological activity and the CD spectrum of C3a. Heat treatment or reduction and alkylation of human C3a produced biologically inactive and conformationally modified anaphylatoxin. In contrast, C3a inactivated by enzymatic removal of the COOH-terminal arginyl residue was structurally unchanged as judged by CD measurements. Consequently, it is proposed that in addition to the essential COOH-terminal arginyl residue, a highly ordered conformation is required for biological functionality of the C3a molecule.

Membrane-active polypeptides as small as angiotensin and bradykinin have been shown to assume ordered conformations in free solution (1, 2). However, conformation has yet to be demonstrated an essential parameter of bradykinin or angiotensin function. C3a1 anaphylatoxin, angiotensin, and bradykinin all induce a number of similar cellular responses through interactions of high specificity at the membrane level. Responses of smooth muscle tissue and mast cells are induced with C3a concentrations as low as 10⁻¹⁰ to 10⁻¹⁰ M. Since the structural basis of anaphylatoxin activity has not been explored herebefore, an analysis was undertaken with the aid of CD to assess: (a) the nature and extent of ordered conformation in the C3a molecule and (b) whether the secondary conformation of C3a is an essential structural requirement for expression of anaphylatoxin activity. It has previously been shown that selective removal of the COOH-terminal arginine inactivates C3a with respect to induction of smooth muscle contraction (3, 4) and histamine release from mast cells (5). Therefore, the CD spectrum of C3a was examined also after removal of the COOH-terminal residue for effects on the conformation of the polypeptide.

EXPERIMENTAL PROCEDURES

Materials—Human and porcine C3a anaphylatoxins were prepared according to the procedures described in the preceding paper (4), and C3 was obtained by the method of Nilsson and Muller-Eberhard (6). Bovine pancreatic carboxypeptidase B (DFP treated) was obtained from Worthington. Ultrapure grade guanidinium chloride (Gdn-HCl) was purchased from Schwarz-Mann. Iodoacetamide was obtained from Calbiochem. Mercaptoethanol was purchased from Eastman. Cellulose casing (small pore, 23 mm) was obtained from Union Carbide and was prepared for use by boiling in sodium bicarbonate (1 g per liter) and EDTA (100 mg per liter) followed by multiple washes with distilled water. Other chemicals used were reagent grade.

Assay for C3a Anaphylatoxin—An assay measuring smooth muscle contraction was performed as described (7). Hem sections were excised from 300- to 400-g guinea pigs and 2- to 3-cm strips were suspended in a 10-ml organ bath. The tissue was bathed at 37° in Tyrode's solution and oxygen was bubbled through the organ chamber. The muscle was tied to a heart/smooth muscle transducer (Harvard Apparatus Co., Catalog No. 350) on line with a strip chart recorder and chart drive (Harvard Apparatus Co., models 480 and 550). The minimum dose of native C3a required to produce a full contraction was determined to be

1 The abbreviations used are: C3a and C3b, products formed by a site-specific enzyme attack on the third component of complement (C3); Gdn-HCl, guanidinium chloride; CpeB, bovine pancreatic carboxypeptidase B.
Various Treatments of C3a Anaphylatoxin—Approximately 2 mg of human C3a were dissolved in 2 ml of 0.1 m sodium phosphate buffer, pH 7.2, and 50 µ of mercaepoethanol were added. Reduction proceeded for 30 min at room temperature in the absence of denaturant. Iodoacetamide (150 mg) was added and the pH was maintained above 7 for 15 min by frequent additions of 0.1 m sodium hydroxide. The entire mixture was transferred to a small cellulose cation and dialyzed in 500 ml of 0.02 m sodium phosphate buffer, pH 7.2, at 4°C with three changes over a 24-hour period. An aliquot was taken for amino acid analysis. Human C3a in 0.02 m phosphate buffer (2 mg per ml) was diluted with 10 m Gdn-HCl to a final concentration of 6 m Gdn-HCl and 0.02 m phosphate at pH 7.2. Another sample of the same C3a preparation was prepared as above to contain 6 m Gdn-HCl and mercaepoethanol was added to a final concentration of 0.02 m. Samples of C3a previously treated with Gdn-HCl or with Gdn-HCl and mercaepoethanol were dialyzed against 0.02 m sodium phosphate at pH 7.2, overnight at 4°C for removal of the denaturants. Digestion of C3a with carboxypeptidase B was performed inside the cuvettes used for the CD measurement in order to minimize manipulative errors. C3a was digested with 0.5% (w/w) CpB in 0.02 m sodium phosphate at pH 7.2 and room temperature for 10 min. Samples were prepared for a pH study by dialyzing separate aliquots of C3a at 4°C against either 0.1 m HCl, 0.15 m sodium acetate at pH 3.7, 0.02 m sodium phosphate at pH 7.2, or 0.02 m ethanolamine at pH 9.6.

Determination of Protein Concentration—Concentrations of all C3a samples examined by CD were estimated by automatic amino acid analysis after 22 hours of acid hydrolysis. Protein concentrations were calculated based on the assumed integral values reported for C3a (4). All analyses were performed according to the methods of Spackman et al. (8) as modified for accelerated analysis (9).

Spectral Measurements—A Cary model 61 recording spectropolarimeter was used for all CD measurements. Spectra were scanned and retraced at 27°C ± 0.2°C with excellent reproducibility at 2 or 6 nm per min and a pen period of 3 to 10 s at full scale settings of 20 or 50 millidegrees. The slit width of the instrument was programmed to yield constant light intensity over the spectral range examined. Dynode voltage never exceeded 600 volts, and was programmed to yield constant light intensity over the spectral settings of 20 or 50 millidegrees. The slit width of the instrument at 2 or 6 nm per min and a pen period of 3 to 10 s at full scale. Scans were nearly identical between 190 and 250 nm and the extrema at 208 and 222 nm were within 5% of complete agreement between the two proteins. These CD spectra closely resemble the spectrum reported for the helical form of poly(L-lysine), as well as the spectra recorded for the proteins papain, insulin, and staphylococcal nuclease (11). Comparison of C3a with synthetic poly(L-lysine) seems justified considering the basic nature of anaphylatoxins. Approximately 25% of the amino acids in human and porcine C3a are basic residues (4). In contrast, the CD spectrum obtained for the parent C3 molecule (see Fig. 1) indicated a smaller relative content of ordered conformation and, in particular, a helical content was much less evident.

Fig. 2 shows the similarity in ultraviolet absorption between the two C3a anaphylatoxins. These spectra are relatively simple because the C3a molecule contains only 2 tyrosyl, 3 phenylalanyl, and no tryptophanyl residues. Absorbance maxima occurred at 276 nm for both the human and porcine C3a, and E%nm values were estimated to be 6.3 and 6.45, respectively. The circular dichroism signals in the same ultraviolet range (250 to 350 nm) were of very low magnitude (e.g., [9] less than ±10 deg cm2 per dmol of protein) and therefore are not shown here.

Effects of pH and Heat on Structure and Function—Since the method for C3a preparation utilized rather acidic conditions, a study was made to ascertain the effects of pH on the conformation of C3a. The CD spectra for human C3a at pH conditions from 1 to 9.6 are illustrated in Fig. 3. Only minor pH-dependent changes were observed and no loss in activity could be detected with the bioassay for any of the pH conditions, including pH 1. However, approximately 20% of the ellipticity at 208 and 222 nm was eliminated in the spectrum of human C3a at pH 1 compared with neutrality. This change of the CD pattern was reversed by dialysis of C3a against buffer of pH 7.2. In general, the magnitude of the C3a ellipticity increased with increasing pH. Similar behavior has been reported for preparations such as poly(L-lysine) the side chains of which are ionized at low pH (12).

Absorption spectra were recorded using a Cary model 118C spectrophotometer with cells of 1.0-cm path length.

**RESULTS**

Native Human and Porcine C3a—The CD spectra obtained for human and porcine C3a are illustrated in Fig. 1. The curves were nearly identical between 190 and 250 nm and the extrema at 208 and 222 nm were within 5% of complete agreement between the two proteins. These CD spectra closely resemble the spectrum reported for the helical form of poly(L-lysine), as well as the spectra recorded for the proteins papain, insulin, and staphylococcal nuclease (11). Comparison of C3a with synthetic poly(L-lysine) seems justified considering the basic nature of anaphylatoxins. Approximately 25% of the amino acids in human and porcine C3a are basic residues (4). In contrast, the CD spectrum obtained for the parent C3 molecule (see Fig. 1) indicated a smaller relative content of ordered conformation and, in particular, a helical content was much less evident.

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The thermal stability of human C3a was examined by heating in 0.02 M sodium phosphate at pH 7.2 and then measuring the CD spectrum and the biological activity at 25°C. No loss of activity could be detected after heating for 30 min at 80°C or for 5 min at 100°C. However, approximately 40% activity loss was recorded when C3a was heated for 30 min at 100°C. After 3 hours at 100°C, C3a had irreversibly lost more than 90% of the activity. As a result, heating at 100°C, however, approximately 40% activity loss was recorded when C3a was heated for 30 min at 100°C. After 3 hours at 100°C, C3a had irreversibly lost more than 90% of the activity. This result shows that C3a was adjusted to pH 3.7 and gel-filtered on a Sephadex G-50 column. The C3a eluted at a position indicating a molecular weight between 9,000 and 10,000. This result shows that C3a remains in a monomeric form following heat denaturation.

Lack of Contribution of COOH terminal Residue to Secondary Structure—The biological activity of both human and porcine C3a anaphylatoxin is abolished by the removal of the COOH-terminal arginyl residue (3, 4). Data presented here (Table I, A, B, and H) affirm the completeness of C3a inactivation by short term digestion with carboxypeptidase B. Cpb-treated C3a migrated as a slightly less basic protein than the native C3a on cellulose acetate strip electrophoresis at pH 8.6 (4). No differences could be discerned between the CD spectra of human C3a before and after Cpb digestion. When Cpb-digested C3a was treated with 0.02 M mercaptoethanol and the reducing agent was removed by dialysis, the CD spectrum was identical with that of intact C3a. These results indicate that the COOH-terminal arginyl residue of C3a fulfills a functional rather than a structural role.

Effects of Guanidinium Chloride and Mercaptoethanol on Structure and Function—Disruption of the structure of human C3a was extensive in 6 M Gdn-HCl as illustrated in Fig. 5 (Curve C). The absolute mean residue ellipticity at 222 nm was reduced by 50%. Absorbance of the guanidino group prevented measurements below 210 nm; therefore conclusions pertaining to relative structural effects were deduced from the 222-nm band. The residual structure of C3a in 6 M Gdn-HCl was constant over several hours of measurement. C3a activity was estimated using material dissolved in 6 M Gdn-HCl, full activity was obtained (see Table I, D). Since activity measurements cannot be made in the presence of 6 M Gdn-HCl, these results can be interpreted in two ways. Either 6 M Gdn-HCl had no effect on C3a function or, more likely, reversible folding had occurred upon dilution of the denaturant in the organ bath. If it occurred, it did so rapidly, since the interval between sample injection and the recording of a response is 5 to 10 s.

When both mercaptoethanol and 6 M Gdn-HCl were added to C3a a complete elimination of the 222-nm CD band was observed (see Fig. 5, Curve A). Functional assays on C3a samples removed from this mixture indicated approximately 90% inactivation (Table I, E). Inactivation was time-dependent; the activity decayed with a half-life of 2 min. The residual activity of C3a in mercaptoethanol and 6 M Gdn-HCl never fell below 10% of

<table>
<thead>
<tr>
<th>Material and treatment</th>
<th>Activitya</th>
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<tbody>
<tr>
<td>A. Human C3a</td>
<td>0.8 to 1.0</td>
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<tr>
<td>B. Porcine C3a</td>
<td>1.0 to 1.2</td>
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<tr>
<td>C. Human C3a in 0.02 M mercaptoethanol</td>
<td>0.8 to 1.0</td>
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<tr>
<td>D. Human C3a in 6 M Gdn-HCl</td>
<td>10 to 12</td>
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<tr>
<td>E. Human C3a in 0.02 M mercaptoethanol and 6 M Gdn-HCl</td>
<td>&gt; 20d</td>
</tr>
<tr>
<td>F. Sample E dialyzed in phosphate, pH 7.2</td>
<td>0.8 to 1.0</td>
</tr>
<tr>
<td>G. Carboxyamidomethyl-C3a (human)</td>
<td>&gt; 20d</td>
</tr>
<tr>
<td>H. Carboxypeptidase-treated human and porcine C3a</td>
<td>&gt; 20d</td>
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</table>

a Activities were determined in a 10-ml organ bath maintained at 37°C using a 2- to 3-cm strip of guinea pig ileum according to Cochrane and Müller-Eberhard (7). Quantities indicate C3a required for a maximum contractile response.

b Activities were obtained following incubation for 90 min at room temperature.

c Sample G was prepared according to the procedure described under "Experimental Procedures." The alkylated C3a contained 5.7 carboxyamidomethyllysine residues per molecule of anaphylatoxin.

d No activity was recorded with 20-μg aliquots of Samples G or H. Neither sample produced desensitization of the muscle strip when challenged with native C3a.

<FIG. 3. Circular dichroism spectra for human C3a under various pH conditions. The individual spectra represent human C3a dissolved in 0.1 M HCl (Curve A); in 0.05 M sodium acetate at pH 3.7 (Curve B); in 0.02 M sodium phosphate at pH 7.2 (Curve C); and in 0.02 M ethanolamine at pH 9.6 (Curve D). All samples contained 0.2 M sodium chloride in an attempt to normalize salt effects.

<FIG. 4. Circular dichroism spectra for human C3a under various pH conditions. The individual spectra represent human C3a dissolved in 0.1 M HCl (Curve A); in 0.05 M sodium acetate at pH 3.7 (Curve B); in 0.02 M sodium phosphate at pH 7.2 (Curve C); and in 0.02 M ethanolamine at pH 9.6 (Curve D). All samples contained 0.2 M sodium chloride in an attempt to normalize salt effects.

<FIG. 5. Circular dichroism spectra for human C3a under various pH conditions. The individual spectra represent human C3a dissolved in 0.1 M HCl (Curve A); in 0.05 M sodium acetate at pH 3.7 (Curve B); in 0.02 M sodium phosphate at pH 7.2 (Curve C); and in 0.02 M ethanolamine at pH 9.6 (Curve D). All samples contained 0.2 M sodium chloride in an attempt to normalize salt effects.
that observed with the native material. This result may reflect the inherent ability of the C3a molecule to refold in the organ bath upon dilution of the denaturing agents. Full activity of C3a was restored by dialysis against pH 7.2 phosphate buffer (see Table I, F). Fig. 5 (Curve D) shows that, after dialysis, the CD spectrum and hence the conformation of the C3a were again identical with native C3a.

Addition of 0.02 M mercaptoethanol to human C3a produced a time-dependent structural change that could be readily monitored at 222 nm (Fig. 6). As was observed with 6 M Gdn-HCl treatment, approximately 50% of the original CD contribution at 222 nm was eliminated by mercaptoethanol, but only after 90 min of incubation at room temperature. Calculations according to the equations of Chen and Yang (13) indicated a 21% helical content in the reduced human C3a after 60 min as compared to 16% residual helix content in the reduced and alkylated anaphylatoxin. The reduced and alkylated C3a contained 5.7 carboxyamidomethylcysteine residues per molecule. An extremum was located at 202 nm indicating a large change in the structure toward a random conformation (see Fig. 4, Curve B). A halftime of 15 min was estimated for the structural transition of C3a in the presence of mercaptoethanol.

Activity data for the reduced and the reduced and alkylated C3a samples are given in Table I, C and G, respectively. C3a activity was not measureably affected by mercaptoethanol due to the irreversible scission of disulfide bonds.

Presumably the concentration of mercaptoethanol is sufficiently reduced during the assay to permit the C3a molecule to refold and exhibit activity. However, when mercaptoethanol is added to the organ bath to a final 0.01 M concentration the responsiveness of smooth muscle to C3a is diminished. Addition of mercaptoethanol had no effect on the smooth muscle contraction induced by histamine. Unlike bradykinin, for which reducing agents actually potentiate smooth muscle contraction upon dilution of the denaturing agents. Full activity of C3a was estimated for the structural transition of C3a.

Discussion

Empirical evaluation of CD spectra for the estimation of the ordered and random structure of proteins has recently been extended to molecules with known secondary conformations (12, 13). Several proteins with three-dimensional structures determined by X-ray crystallography have been examined by CD. A favorable correlation was obtained between the fractional content of helix determined in the crystalline state and the respective CD contributions at 222 nm for a number of proteins in solution (13). Similarly, empirical equations were derived from the ellipticity at 208 nm obtained with model polyamino acids for application to proteins (12). In the present study, values of 41 to 43 and 42 to 45% helix were obtained for human and porcine C3a, respectively, which are based on empirical equations for ellipticity at 222 nm (13). In comparison, a 41 to 46% helical content was estimated for both human and porcine C3a based on a poly(L-lysine) reference at 208 nm (12). Regardless of the reference or spectral bands employed for these estimates, the most apparent structural feature of C3a clearly is an abundance of $\alpha$ helix. On the other hand, the parent C3 molecule contains relatively little of either $\alpha$ helical or $\beta$ structure according to CD measurements.

A striking property of the C3a molecule is the capacity to refold following extensive denaturation. Reduction of the disulfide bonds in C3a produced a slow unfolding of the molecule, but had no detectable effect on the biological activity, probably because refolding proceeded rapidly on removal of the reducing agent. However, it was shown that one or all of the disulfide linkages in C3a are required for maintaining the structural and functional integrity of the molecule. Alkylation of reduced C3a produced a completely inactive derivative which had undergone structural disruption similar to that caused by reduction alone. Neither guanidinium chloride nor mercaptoethanol alone were capable of totally unfolding the anaphylatoxin. Only when both agents were employed was the existing secondary conformation apparently converted to a random coil. Although C3a refolded upon removal of these agents, refolding was sufficiently slow to allow detection of a sizeable effect on the biological activity. These observations together with the demonstration of irreversi-
ble heat inactivation clearly indicate the importance of secondary conformation to the functionality of C3a.

Enzymatic removal of the COOH-terminal arginine of human C3a produced no apparent changes in CD spectrum. Absence of detectable changes in the secondary conformation of C3a after the biological activity had been abolished by the action of carboxypeptidase B implies a functional rather than a structural role of the terminal arginyl residue. It may be concluded, therefore, that the activity of C3a depends on the conformational integrity of the molecule as well as the primary structure of the COOH-terminal region.

The fact that the original conformation of C3a was restored after the molecule had been extensively unfolded supports the thermodynamic hypothesis of spontaneous and reversible folding of polypeptides (15). However, C3a anaphylatoxin is not synthesized as such, rather it is a proteolytic product representing only 4 to 5% of the native C3 molecule. Consequently, either a structural rearrangement in the C3a moiety has occurred immediately following release from the C3 molecule or the C3a exists in a highly ordered and stable conformation within the covalent structure of the parent C3 molecule. Although the mean residue ellipticity was lower for C3 than for C3a, the absolute CD contribution for the larger C3 molecule in the regions of 208 and 222 nm was much greater than that measured for the isolated C3a portion. In addition, three disulfide bonds are involved in the refolding of reduced C3a and therefore the latter hypothesis appears most likely to be the case. The C3a portion of C3 may therefore represent a model for the concept advanced by Anfinsen (15) and Wetlaufer (16) in that it may constitute a local region of detectable changes in the secondary conformation of C3a after the molecule had been extensively unfolded supports the thermodynamic hypothesis of spontaneous and reversible folding of polypeptides (15). However, C3a anaphylatoxin is not synthesized as such, rather it is a proteolytic product representing only 4 to 5% of the native C3 molecule. Consequently, either a structural rearrangement in the C3a moiety has occurred immediately following release from the C3 molecule or the C3a exists in a highly ordered and stable conformation within the covalent structure of the parent C3 molecule. Although the mean residue ellipticity was lower for C3 than for C3a, the absolute CD contribution for the larger C3 molecule in the regions of 208 and 222 nm was much greater than that measured for the isolated C3a portion. In addition, three disulfide bonds are involved in the refolding of reduced C3a and therefore the latter hypothesis appears most likely to be the case. The C3a portion of C3 may therefore represent a model for the concept advanced by Anfinsen (15) and Wetlaufer (16) in that it may constitute a local region which is capable of providing nucleation for folding of the entire C3 molecule. It is known that the membrane binding capacity of activated C3 (C3b) decays rapidly once C3 has been cleaved and thus result in a spontaneous relaxation of the C3b molecule. It has been reported that dissociation constants at the membrane level are of the order of 10^{-19} M for several bioactive peptides including insulin (18), nerve growth factor (19), and oxytocin (20) based on the concentrations at which these agents function (21). C3a would seem to possess a similar dissociation constant since cellular responses to C3a have been recorded at concentrations in the same range as those observed for hormonal action. Further characterization of the secondary structure of C3a will aid in describing the high affinity interactions which afford attachment to membrane receptors. Knowing that explicit conformational requirements are involved in the expression of C3a activity may facilitate formulation of a conceptual model for an effector-receptor complex for the anaphylatoxin.

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